

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 041673/2036	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 99/ 22107	International filing date (day/month/year) 24/09/1999	(Earliest) Priority Date (day/month/year) 25/09/1998
Applicant THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 22107

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 99/22107

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/102 A61K39/116

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANG Y F ET AL: "Apoptosis: a possible tactic of Haemophilus somnus for evasion of killing by bovine neutrophils?" MICROBIAL PATHOGENESIS, (1998 JUN) 24 (6) 351-9. , XP000891692 ---	1,3,4
A	GOGOLEWSKI, RONALD P. ET AL: "Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of Haemophilus somnus" INFECT. IMMUN. (1988), 56(9), 2307-16 , XP002137019 page 2308, column 1, paragraph 1 page 2315, column 2, paragraph 1 --- -/--	3,6,9,10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

4 May 2000

Date of mailing of the international search report

18/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Charles, D



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/22107

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	CORBEIL L B ET AL: "Characterization of immunodominant surface antigens of Haemophilus somnus." INFECTION AND IMMUNITY, (1991 DEC) 59 (12) 4295-301. , XP002137020 cited in the application page 4295, column 1, paragraph 2 page 4300, column 1, paragraph 2 -----	3,6,9,10
A	US 4 981 685 A (M.C. HEALEY) 1 January 1991 (1991-01-01) column 2, line 60 -column 3, line 25; claim 1; example 30 -----	3,6,9,10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/22107

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



Information on patent family members

PCT/US 99/22107

Form PCT/ISA/210 (patent family annex) (July 1992)



PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

TAYLOR, Stacy L.
FOLEY & LARDNER
401 West Broadway
Suite 23
San Diego, CA 92101-3542
ETATS-UNIS D'AMERIQUE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence
and Administrative Instructions, Section 601(a))

PER TELEFAX : 18.05.00

Date of mailing
(day/month/year)

23.05.00

Applicant's or agent's file reference

041673/2036-2039

IMPORTANT NOTIFICATION

International application No.

PCT/US 99/ 22107

International filing date (day/month/year)

24/09/1999

Priority date (day/month/year)

25/09/1998

Applicant

THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

25/04/2000

COMPUTER UPDATED
RECEIVED
JUN 01 2000

2. This date of receipt is:

- ☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- ☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- ☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

- ☐ (If applicable) This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. (+ 49-89) 2399-0, Tx: 523656 epmu d
Fax: (+ 49-89) 2399-4465

Authorized officer

AITKEN J M

Tel. (+ 49-89) 2399-2735





PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To

TAYLOR, Stacy L.
FOLEY & LARDNER
401 West Broadway
Suite 23
San Diego, CA 92101-3542
ETATS-UNIS D'AMERIQUE

PCT

WRITTEN OPINION

(PCT Rule 66)

Applicant's or agent's file reference 041673/2036		Date of mailing (day/month/year) 11.09.2000
International application No. PCT/US99/22107		REPLY DUE within 3 month(s) from the above date of mailing
International filing date (day/month/year) 24/09/1999	Priority date (day/month/year) 25/09/1998	
International Patent Classification (IPC) or both national classification and IPC A61K39/00		
Applicant THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.		

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain document cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

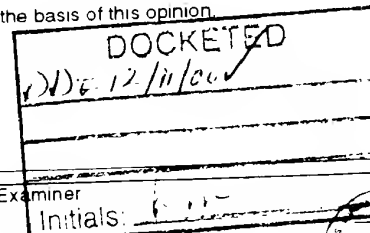
When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3
For the form and the language of the amendments, see Rules 66.8 and 66.9

Also: For an additional opportunity to submit amendments, see Rule 66.4
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: **25/01/2001**



Name and mailing address of the international preliminary examining authority



European Patent Office
D-80298 Munich
Tel +49 89 2399 - 0 Tx 523656 epmu d
Fax +49 89 2399 - 4465

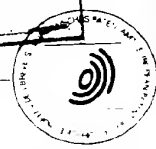
Authorized officer / Examiner

Weijland, A

Formalities officer (incl. extension of time limits)

Danti, B

Telephone No +49 89 2399 8161





WRITTEN OPINION

International application No. PCT/US99/22107

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-15 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/1 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

- ☒ the entire international application,
- ☐ claims Nos. .

because:

- ☒ the said international application, or the said claims Nos. 1-13 (with respect to industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):



WRITTEN OPINION

International application No. PCT/US99/22107

see separate sheet

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1, 5, 6, 9, 10 No
Inventive step (IS)	Claims	1, 5, 6, 9-11 No, 2-4, 7, 8, 12, 13 ?
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet



The following documents (D) are referred to in this opinion; the numbering will be adhered to the rest of the procedure:

- D1: US-A-4 981 685 (M.C. HEALEY) 1 January 1991 (1991-01-01)
D2: CORBEIL L B ET AL: 'Characterization of immunodominant surface antigens of *Haemophilus somnus*.' INFECTION AND IMMUNITY, (1991 DEC) 59 (12) 4295-301.

SECTION III

1. For the assessment of the present claims 1-13 on the question whether they are industrially applicable, no unified criteria exist in the PCT contracting states. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in a medical treatment and the use of such compound for the manufacture of a medicament for new medical treatment.

In the above mentioned context the passage in claim 1 "A method for vaccinating cattle against diseases" is considered to cover treatment by therapy.

Therefore, claims 1-13 relate to the subject-matter considered by this authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

SECTION V

2. The subject matter of claims 1, 5, 6, 9-10 is not novel (Article 33(2) PCT).

- 2.1 Claims 1, 5, 6, 9 and 10 are anticipated by D1.

D1 (claim 2) describes a method for immunizing sheep ("a method for vaccinating cattle... vaccine" according to present claim 1) comprising *H. somnus* bacteria which are administered in an immunologically effective amount. The bacteria are



contacted with a detergent to extract antigens from the outer membrane ("expresses protective antigen", "40 kDa outer membrane protein" according present claims 9 and 10), without denaturation of the antigen ("*H. somnus* is live", or "killed" according to present claims 5, 6).

2.2 Claims 1, 9 and 10 are anticipated by D2. D2 (abstract) describes the cross reactivity of antiserum to p40 with antigens of members of the family *Pasteurellaceae* and the ability of this antiserum to protect against *H. somnus* pneumonia indicate that p40 may be a useful vaccine antigen ("A method for vaccinating cattle...vaccine" according to present claim 1, "protective antigen" according to claim 9, "40kDa outer membrane protein " according to claim 10) for *H. somnus* disease. The strains 1P and 129Pt are disclosed.

2.3 The subject matter of claims 2-5, 7, 8, 11-13 is novel.

Claims 2-5, 7, 8, 11-13, relating to methods for vaccinating cattle, is not disclosed in the prior art documents.

3. Inventive Step (Article 33(3) PCT)

3.1 At present the subject-matter of claims 2-5, 7, 8, 11-13, cannot be definitely assessed as regards inventive step. Mere allegations as regards the "effective amount of an *H. somnus* vaccine" cannot be accepted at face value.

The applicant is however, reminded of the fact that any information he may wish to submit concerning the subject-matter of the invention, for example further details of its advantages or of the problem it solves, and for which there is no basis in the application as filed, should be confined to the letter of reply rather than incorporated into the application, cf. Article 34(2)(b) PCT.

3.2 Should the applicant be able to overcome the afore mentioned objection, the presence of an inventive step could be acknowledged for the subject-matter of claims 2-5, 7, 8, 11-13 (Article 33(3) PCT).

The closest state of the art is considered to result from D1. Claims 2-5, 7, 8, 11-13



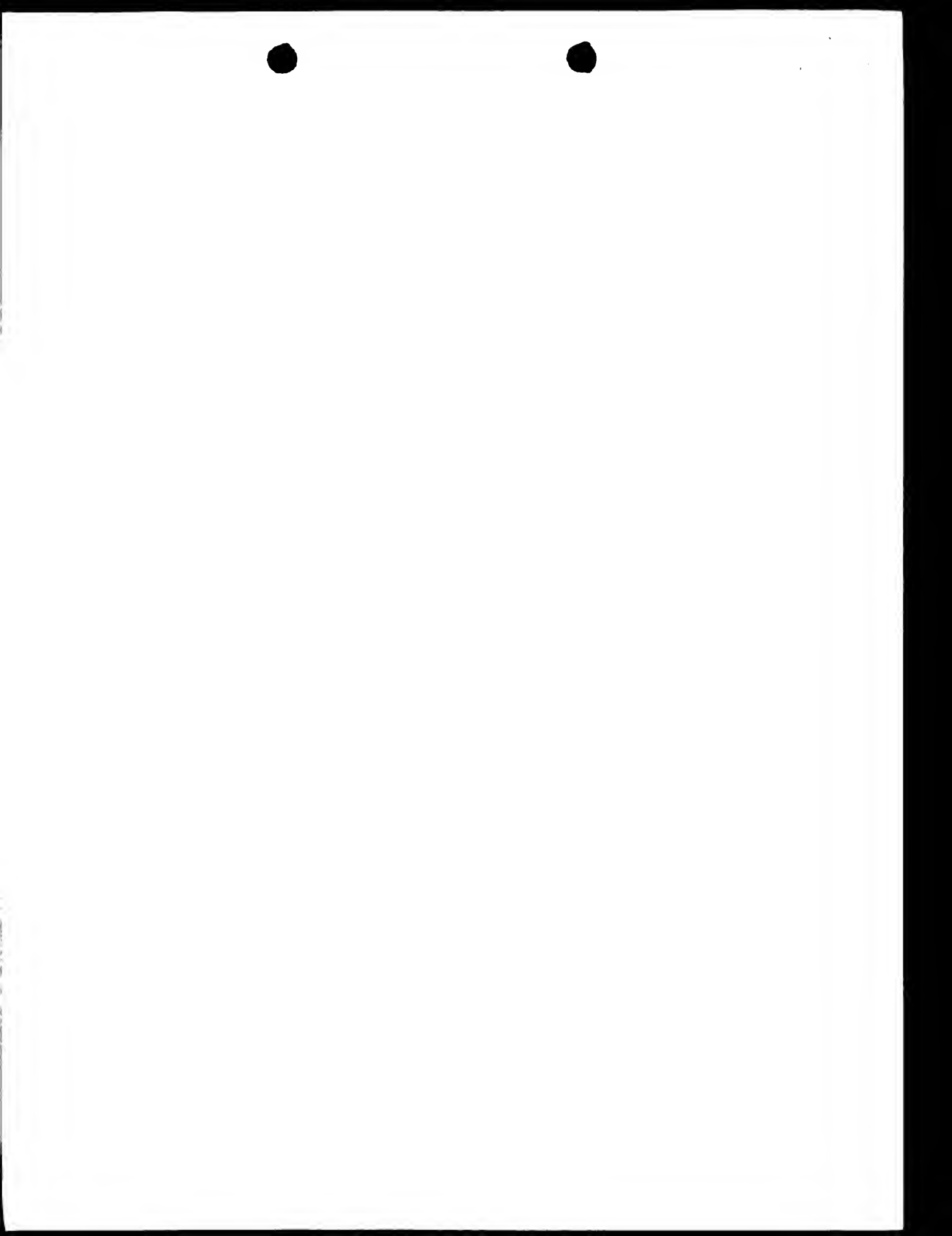
differ from D1 in that these claims describe certain modified *H. somnus* strains. These strains have improved characteristics to be used as a vaccine, they produce reduced amounts of endotoxin (claims 2-4) or of immunoglobulin binding proteins (claims 7, 8) or produce more protective antigens (claims 11-13). There is not hint in the prior art documents that these characteristics could lead to an improved vaccine.

SECTION VII

4. The phrase "and incorporated by reference..." as mentioned e.g. on page 15 (line 11-14) contravenes the requirement that the application needs to be self contained (see further Guidelines C-II 4.17).
5. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in D1 and D2 is not mentioned in the description, nor are these documents identified therein.

SECTION VIII

6. The subject matter of claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not defined. Claim 1 attempts to define the subject matter in terms of results to be achieved. Such formulations are not allowable, because it appears possible to define the subject matter in more concrete terms, viz. in terms of how the effect, i.e. effective vaccine, is to be achieved (see the technical features in claims 2, 3, 7-13).
7. The term "effective amount" is not clear and contravenes the requirements of Article 6 PCT.





PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 041673/2036		FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/22107		International filing date (<i>day/month/year</i>) 24/09/1999		Priority date (<i>day/month/year</i>) 25/09/1998
International Patent Classification (IPC) or national classification and IPC A61K39/00				
Applicant THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.				
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 7 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>				
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input checked="" type="checkbox"/> Certain defects in the international application VIII <input checked="" type="checkbox"/> Certain observations on the international application 				
Date of submission of the demand 25/04/2000		Date of completion of this report 30.01.2001		
Name and mailing address of the international preliminary examining authority.  European Patent Office D-80298 Munich Tel +49 89 2399 - 0 Tx 523656 epmu d Fax +49 89 2399 - 4465		Authorized officer Weijland, A Telephone No. +49 89 2399 7490 		



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/22107

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*

Description, pages:

1-15 as originally filed

Claims, No.:

1-13 as originally filed

Drawings, sheets:

1/1 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/US99/22107

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☒ the entire international application.

☐ claims Nos. .

because:

☒ the said international application, or the said claims Nos. 1-13 (with respect to industrial applicability), 2-4,7,8,12,13 (with respect to inventive step) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/22107

Novelty (N)	Yes:	Claims	2-4,7,8,12,13
	No:	Claims	1, 5, 6, 9, 10
Inventive step (IS)	Yes:	Claims	2-4,7,8,12,13
	No:	Claims	1, 5, 6, 9, 10
Industrial applicability (IA)	Yes:	Claims	1-13?
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet



The following documents (D) are referred to in this opinion; the numbering will be adhered to the rest of the procedure:

D1: US-A-4 981 685 (M.C. HEALEY) 1 January 1991 (1991-01-01)

D2: CORBEIL L B ET AL: 'Characterization of immunodominant surface antigens of *Haemophilus somnus*.' INFECTION AND IMMUNITY, (1991 DEC) 59 (12) 4295-301.

SECTION III

1. For the assessment of the present claims 1-13 on the question whether they are industrially applicable, no unified criteria exist in the PCT contracting states. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in a medical treatment and the use of such compound for the manufacture of a medicament for new medical treatment.

In the above mentioned context the passage in claim 1 "A method for vaccinating cattle against diseases" is considered to cover treatment by therapy.

Therefore, claims 1-13 relate to the subject-matter considered by this authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

SECTION V

2. The subject matter of claims 1, 5, 6, 9-10 is not novel (Article 33(2) PCT).

- 2.1 Claims 1, 5, 6, 9 and 10 are anticipated by D1.

D1 (claim 2) describes a method for immunizing sheep ("a method for vaccinating cattle... vaccine" according to present claim 1) comprising *H.somnus* bacteria which are administered in an immunologically effective amount. The bacteria are



contacted with a detergent to extract antigens from the outer membrane ("*H.somnus* is susceptible to killing by bovine complement containing serum", "expresses protective antigen", "40 kDa outer membrane protein" according present claims 1, 9 and 10), without denaturation of the antigen ("*H.somnus* is live", or "killed" according to present claims 5, 6).

- 3.2 Claims 1, 9 and 10 are anticipated by D2. D2 (abstract) describes the cross reactivity of antiserum to p40 with antigens of members of the family *Pasteurellaceae* and the ability of this antiserum to protect against *H.somnus* pneumonia indicate that p40 may be a useful vaccine antigen ("A method for vaccinating cattle...vaccine" , "*H.somnus* is susceptible to killing by bovine complement containing serum" according to present claim 1, "protective antigen" according to claim 9, "40kDa outer membrane protein" according to claim 10) for *H.somnus* disease. The strains 1P and 129Pt are disclosed.

- 3.3 The subject matter of claims 2-4, 7, 8, 11-13 is novel.

Claims 2-4, 7, 8, 11-13, relating to methods for vaccinating cattle in which *H.somnus* releases reduced amounts of vaccine (claims 2-4) or lacks expression of one or more immunoglobulins (claims 7, 8) or is a natural isolate (claim 11) or is genetically engineered (claims 12 and 13), is not disclosed in the prior art documents.

4. The subject-matter of claims 2-4, 7, 8, 11-13 would appear to involve an inventive step (Article 33(3) PCT).

The closest state of the art is considered to result from D1. Claims 2-4, 7, 8, 11-13 differ from D1 in that these claims describe certain modified *H.somnus* strains. These strains have improved characteristics to be used as a vaccine, they produce reduced amounts of endotoxin (claims 2-4) or of immunoglobulin binding proteins (claims 7, 8) or produce more protective antigens (claims 11-13). There is no hint in the prior art documents that these characteristics could lead to an improved vaccine.

SECTION VII



5. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in D1 and D2 is not mentioned in the description, nor are these documents identified therein.

SECTION VIII

6. The term "reduced amounts" in claims 2 and 3 is not clear in defining the range of endotoxin and therefore contravenes Article 6 PCT.



IPEA/ EP

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:

The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND
------------------------	---------------------------

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference
International application No. PCT/US99/22107	International filing date (day/month/year) 24 September 1999	(Earliest) Priority date (day/month/year) 25 September 1998

Title of invention

VACCINE BASED ON ATTENUATED HAEMOPHILUS SOMNUS

Box No. II APPLICANT(S)		
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) THE REGENTS OF THE UNIVERSITY OF CALIFORNIA Office of Technology Transfer 1111 Franklin Street, 5 th Floor UNITED STATES OF AMERICA	Telephone No.:	
	Facsimile No.:	
	Teleprinter No.:	
State (i.e., country) of nationality:	State (i.e., country) of residence:	

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) CORBEIL, Lynette B. 1648 Neale Street San Diego, CA 92103 UNITED STATES OF AMERICA	
State (i.e., country) of nationality: Canada	State (i.e., country) of residence: US

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) ZIEGLER, Elizabeth J. 930 Gage Drive San Diego, CA 92106-2963 UNITED STATES OF AMERICA	
State (i.e., country) of nationality: US	State (i.e., country) of residence: US

<input checked="" type="checkbox"/> Further applicants are indicated on a continuation sheet.



Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet is not to be included in the demand.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SANDERS, Jerry D.
12345 Alcoy Drive
Fenton, MI 48430
UNITED STATES OF AMERICA

State (i.e. country) of nationality: US

State (i.e. Country) of residence: US

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State (i.e. country) of nationality:

State (i.e. Country) of residence:

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State (i.e. country) of nationality:

State (i.e. Country) of residence:

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

State (i.e. country) of nationality:

State (i.e. Country) of residence:



Further applicants are indicated on another continuation sheet.



No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative
 and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.
☐ is hereby appointed and any earlier appointment of (an) agents/common representative is hereby revoked.
☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

TAYLOR, Stacy L.
 Foley & Lardner
 401 West Broadway, Suite 23
 San Diego, CA 92101-3542
 UNITED STATES OF AMERICA

Telephone No.:
 (619) 234-6655

Facsimile No.:
 (619) 234-3202

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

- (i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.
 (ii) ☐ to take into account the amendments under Article 34 of
 ☐ the description (amendments attached).
 ☐ the claims (amendments attached).
 ☐ the drawings (amendments attached).
 (iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).
 (iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.
 (v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible States *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above)



Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

- | | | |
|--|---|--------|
| 1. amendments under Article 34 | | |
| description | : | sheets |
| claims | : | sheets |
| drawings | : | sheets |
| 2. letter accompanying amendments under Article 34 | : | sheets |
| 3. copy of amendments under Article 19 | : | sheets |
| 4. copy of statement under Article 19 | : | sheets |
| 5. other (specify): | : | sheets |

For International Preliminary Examining Authority use only

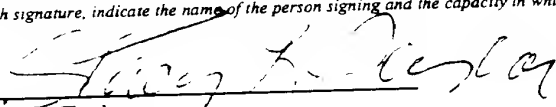
received	not received
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>
<input type="checkbox"/>	<input type="checkbox"/>

The demand is also accompanied by the item(s) marked below:

- | | |
|--|--|
| 1. <input type="checkbox"/> separate signed power of attorney | 4. <input checked="" type="checkbox"/> fee calculation sheet |
| 2. <input type="checkbox"/> copy of general power of attorney | 5. <input type="checkbox"/> other (specify): |
| 3. <input type="checkbox"/> statement explaining lack of signature | |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

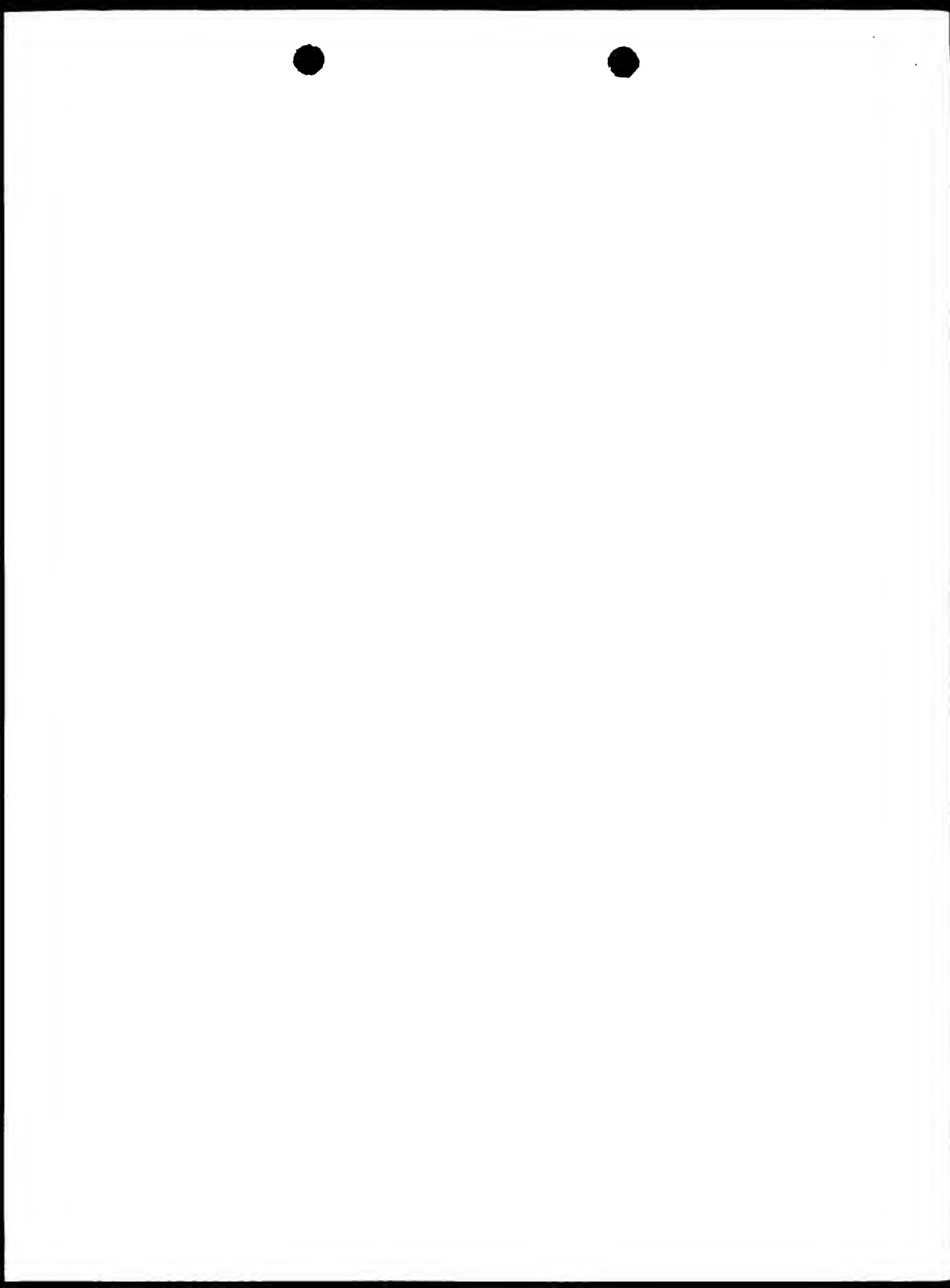

 Stacy L. Taylor
 Attorney for Applicants

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:	
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):	
3. <input type="checkbox"/> The date of receipt of the demand AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.	<input type="checkbox"/> The applicant has been informed accordingly.
4. <input type="checkbox"/> The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5	
5. <input type="checkbox"/> Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.	

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Demand received from IPEA on:

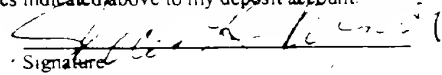


PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

For International Preliminary Examining Authority use only

International application No.	PCT/US99/22107	Date stamp of the IPEA
Applicant's or agent's file reference	041673/2039	
Applicant THE REGENTS OF THE UNIVERSITY OF CALIFORNIA		
Calculation of prescribed fees		
1. Preliminary examination fee	750.00	
2. Handling fee	153.00	
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	903.00	
		TOTAL
Mode of Payment		
<input type="checkbox"/> authorization to charge deposit account with the IPEA (see below)		<input type="checkbox"/> cash
<input checked="" type="checkbox"/> cheque		<input type="checkbox"/> revenue stamps
<input type="checkbox"/> postal money order		<input type="checkbox"/> coupons
<input type="checkbox"/> bank draft		<input type="checkbox"/> other (specify):
Deposit Account Authorization (this mode of payment may not be available at all IPEAs)		
The IPEA/ EP <input type="checkbox"/> is hereby authorized to charge the total fees indicated above to my deposit account.		
<input checked="" type="checkbox"/> (this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit) is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.		
50-0872	25 April 2000	 Signature
Deposit Account Number	Date (day/month/year)	





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/102, 39/116	A3	(11) International Publication Number: WO 00/18429 (43) International Publication Date: 6 April 2000 (06.04.00)
(21) International Application Number: PCT/US99/22107 (22) International Filing Date: 24 September 1999 (24.09.99) (30) Priority Data: 60/101,760 25 September 1998 (25.09.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/101,760 (CIP) Filed on 25 September 1998 (25.09.98) (71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; Office of Technology Transfer, 5th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CORBEIL, Lynette, B. [CA/US]; 1648 Neale Street, San Diego, CA 92103 (US). ZIEGLER, Elizabeth, J. [US/US]; 930 Gage Drive, San Diego, CA 92106-2963 (US). SANDERS, Jerry, D. [US/US]; 12345 Alcoy Drive, Fenton, MI 48430 (US).		(74) Agents: WILSON, Barry, S. et al.; Foley & Lardner, Suite 23, 401 West Broadway, San Diego, CA 92101-3542 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 6 July 2000 (06.07.00)
(54) Title: VACCINE BASED ON ATTENUATED <i>HAEMOPHILUS SOMNUS</i>		
(57) Abstract <p>The present invention provides a method for protecting cattle from diseases such as septicemia, pneumonia or abortion by immunizing them with an <i>H. somnus</i> vaccine. Provided are natural isolates of <i>H. somnus</i> strains that have one or more important features of such vaccine, including, sensitivity to killing in complement-containing bovine serum, lack of expression of immunoglobulin binding proteins, expression of protective antigens and a reduction in the release of endotoxin during growth. Vaccines using <i>H. somnus</i> having these and other features also can be prepared from natural isolates of asymptomatic carriers or from pathogenic organisms by recombinant DNA techniques.</p>		



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EE	Estonia						



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/22107

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/102 A61K39/116

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANG Y F ET AL: "Apoptosis: a possible tactic of Haemophilus somnus for evasion of killing by bovine neutrophils?" MICROBIAL PATHOGENESIS, (1998 JUN) 24 (6) 351-9. , XP000891692	1,3,4
A	GOGOLEWSKI, RONALD P. ET AL: "Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of Haemophilus somnus" INFECT. IMMUN. (1988), 56(9), 2307-16 , XP002137019 page 2308, column 1, paragraph 1 page 2315, column 2, paragraph 1 --- -/-	3,6,9,10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

4 May 2000

Date of mailing of the international search report

18/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Charles, D



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/22107

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CORBEIL L B ET AL: "Characterization of immunodominant surface antigens of Haemophilus somnus." INFECTION AND IMMUNITY, (1991 DEC) 59 (12) 4295-301. , XP002137020 cited in the application page 4295, column 1, paragraph 2 page 4300, column 1, paragraph 2 -----	3,6,9,10
A	US 4 981 685 A (M.C. HEALEY) 1 January 1991 (1991-01-01) column 2, line 60 -column 3, line 25; claim 1; example 30 -----	3,6,9,10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 22107

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



INTERNATIONAL SEARCH REPORT
information on patent family members

International Application No

PCT/US 99/22107

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4981685 A	01-01-1991	NONE	

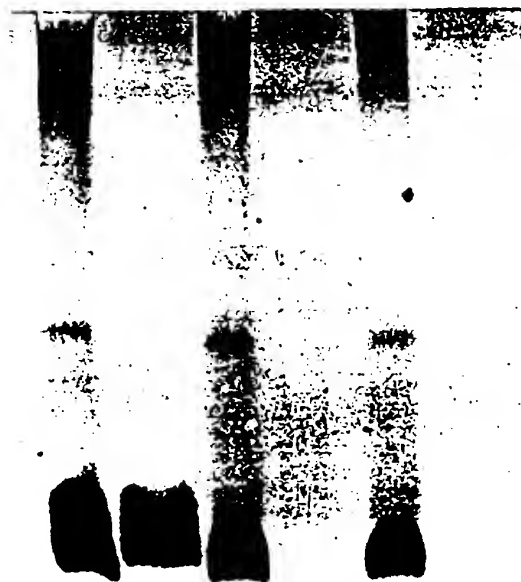




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/00	A2	(11) International Publication Number: WO 00/18429 (43) International Publication Date: 6 April 2000 (06.04.00)
(21) International Application Number: PCT.US99/22107 (22) International Filing Date: 24 September 1999 (24.09.99) (30) Priority Data: 60/101,760 25 September 1998 (25.09.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/101,760 (CIP) Filed on 25 September 1998 (25.09.98) (71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; Office of Technology Transfer, 5th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CORBEIL, Lynette, B. [CA/US]; 1648 Neale Street, San Diego, CA 92103 (US). ZIEGLER, Elizabeth, J. [US/US]; 930 Gage Drive, San Diego, CA 92106-2963 (US). SANDERS, Jerry, D. [US/US]; 12345 Alcoy Drive, Fenton, MI 48430 (US).		(74) Agents: WILSON, Barry, S. et al.; Foley & Lardner, Suite 23, 401 West Broadway, San Diego, CA 92101-3542 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: VACCINE BASED ON ATTENUATED <i>HAEMOPHILUS SOMNUS</i> (57) Abstract The present invention provides a method for protecting cattle from diseases such as septicemia, pneumonia or abortion by immunizing them with an <i>H. somnus</i> vaccine. Provided are natural isolates of <i>H. somnus</i> strains that have one or more important features of such vaccine, including, sensitivity to killing in complement-containing bovine serum, lack of expression of immunoglobulin binding proteins, expression of protective antigens and a reduction in the release of endotoxin during growth. Vaccines using <i>H. somnus</i> having these and other features also can be prepared from natural isolates of asymptomatic carriers or from pathogenic organisms by recombinant DNA techniques.		

2336 129Pt 1P



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VACCINE BASED ON ATTENUATED *HAEMOPHILUS SOMNUS*

This research was supported by funding from the United States Department of
5 Agriculture. Accordingly, the United States may have rights in the invention.

BACKGROUND OF THE INVENTION

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The present invention relates generally to the prevention of diseases of cattle and, more specifically, to immunizing against such diseases by vaccination.

Bovine respiratory disease (BRD), bovine septicemia and bovine reproductive failure (BRF) result in great economic loss to the cattle industry. The primary bacterial
15 pathogens implicated in BRD are *Pasteurella haemolytica*, *P. multocida* and *Haemophilus somnus*. *H. somnus* also causes bovine reproductive failure (BRF) and septicemia.

Current vaccines for *H. somnus* consist mainly of killed bacteria (bacterins) or bacterial extracts. Although there is evidence for protection in some controlled laboratory or animal challenge studies, efficacy in field studies is generally lacking. In some cases the
20 vaccines cause such adverse side effects that their use is very limited. In other cases, little protection is seen. Thus, there is a need to develop improved vaccines to protect cattle from *H. somnus* mediated diseases. Such vaccines should contain key protective antigens that elicit appropriate antibody and cell-mediated immune responses. In addition, such vaccines should lack factors that cause adverse reactions and enable pathogens to evade
25 immune recognition or effector mechanisms.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide an effective and safe
30 *H. somnus* vaccine for protection against BRD, BRF, septicemia and related disorders.

To accomplish these and other objectives, there has been provided, in accordance with one aspect of the present invention, a method for vaccinating cattle against diseases mediated by infection, comprising administering an effective amount of an *H. somnus* vaccine, wherein the *H. somnus* is susceptible to killing by bovine complement-containing
35 serum.

According to another embodiment of the present invention, the *H. somnus* is live.

According to another embodiment of the present invention, the *H. somnus* is killed.

According to yet another embodiment of the present invention, the *H. somnus* lacks the expression of one or more immunoglobulin binding proteins present in virulent *H.*

5 *somnus*. In a further embodiment, the lack of expression of one or more immunoglobulin binding proteins is achieved by the step of genetically engineering *H. somnus* to delete genes encoding the immunoglobulin binding proteins.

According to still yet another embodiment of the present invention, the *H. somnus* expresses a protective antigen. In a further embodiment, the protective antigen is a 40 kDa
10 outermembrane protein.

According to another embodiment of the present invention, the *H. somnus* releases reduced amounts of endotoxin during growth as compared to virulent *H. somnus*.

According to yet another embodiment of the present invention, the *H. somnus* is selected from the group consisting of PTA-600, PTA-601, PTA-602 and PTA-603, all on
15 deposit with the American Type Culture Collection.

In accordance with another aspect of the present invention, a method is provided for vaccinating cattle against diseases mediated by infection, comprising administering an effective amount of an *H. somnus* vaccine, wherein the *H. somnus* releases reduced amounts of endotoxin as compared to virulent *H. somnus*.

20 According to another embodiment of the present invention, the *H. somnus* is live.

According to another embodiment of the present invention, the *H. somnus* is killed.

According to yet another embodiment of the present invention, the *H. somnus* is sensitive to killing by complement-containing bovine serum.

According to still yet another embodiment of the present invention, the *H. somnus*
25 lacks the expression of one or more immunoglobulin binding proteins present in virulent *H. somnus*. In a further embodiment, the lack of expression of one or more immunoglobulin binding proteins is achieved by the step of genetically engineering *H. somnus* to delete genes encoding the immunoglobulin binding proteins.

According to another embodiment of the present invention, the *H. somnus* expresses
30 a protective antigen. In a further embodiment, the protective antigen is a 40 kDa outermembrane protein.

According to yet another embodiment of the present invention, the *H. somnus* is selected from the group consisting of PTA-600, PTA-601, PTA-602 and PTA-603, all on deposit with the American Type Culture Collection.

In further embodiments of the present invention, the vaccines described above use an *H. somnus* genetically engineered to express one or more protective antigens. In further
5 embodiments, the protective antigens are from bacterial pathogens other than *H. somnus*.

Other objects, features and advantages of the present invention will become apparent from the following detailed description.

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BRIEF DESCRIPTION OF DRAWINGS

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Figure 1 is an SDS-polyacrylamide gel showing lipooligosaccharide (LOS), also known as endotoxin, associated with cells or released into media during growth of a virulent *H. somnus* strain (2336) or avirulent *H. somnus* natural isolates (129Pt and 1P). Organisms grown in brain heart infusion broth containing 0.1 % Tris base and 0.01 % thiamine monophosphate were shaken at 37°C. At 24 hours, cultures were adjusted to 75 % light transmission (610 nm) and a cell pellet (CP) was separated from the supernatant (S) by centrifugation. CP and S were digested with RNase followed by proteinase K. After electrophoresis, the gel was silver-stained. Virtually no released LOS could be detected in the S of the avirulent *H. somnus* natural isolates, while the amount of LOS in the CP of both natural isolates and virulent strain of *H. somnus* was similar.

DETAILED DESCRIPTION OF THE INVENTION

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The present invention provides methods for protecting cattle against diseases including, for example, bovine respiratory disease (BRD), bovine septicemia and bovine reproductive failure (BRF), thrombotic meningoencephalitis, arthritis, myocarditis (Gogolewski et al., *Infect. Immun.* 56:2307-2316 (1989); Gogolewski et al., *J. Clin. Microbiol.* 27:1767-1774 (1988); Harris et al., *Can. Vet. J.* 30:816-822 (1989) and Van Donkersgoed et al., *Can. Vet. J.* 35:239-241 (1994)) by immunizing the cattle with an *H. somnus* vaccine. For this purpose, the present invention provides *H. somnus* strains 1P, 129Pt, 130Pfl and 133P, isolated from prepuce of normal bulls and deposited with the

American Type Culture Collection as PTA-600, PTA-601, PTA-602 and PTA-603, respectively, on September 1, 1999.

These "natural" isolates of *H. somnus* are particularly suitable for use in the vaccine method of the present invention because they have several important features. These
5 include, for example, sensitivity to killing in complement-containing bovine serum, lack of expression of immunoglobulin binding proteins, expression of protective antigens and a reduction in the release of endotoxin during growth. The present invention is not limited to such natural isolates. A useful vaccine can include *H. somnus* natural isolates that have less than all the above listed features as well as pathogenic organisms modified so as to share
10 one or more of the unique features associated with the natural isolates. *H. somnus* organisms with such features can be obtained by isolation from natural sources or from diseased tissue. In addition, as discussed further below, useful features for a vaccine can be introduced into by using recombinant DNA techniques to modify *H. somnus*.

One feature of an effective vaccine comprising *H. somnus* is sensitivity to killing in
15 complement-containing bovine serum. *H. somnus* organisms with this feature can be isolated from preputial sites of clinically normal cattle (i.e., asymptomatic carriers) by standard methods (Corbeil et al., *J. Clin. Microbiol.* 22:192-198 (1985)). Such organisms are considered "serum sensitive." Alternatively, the feature of serum sensitivity can be introduced into wildtype or virulent organisms by deleting genes encoding for
20 immunoglobulin binding proteins. Gene deletion methods useful for this purpose, such as homologous recombination, are well known in the art (see Example 2(d)). Thus, the present methods include use of a vaccine comprising *H. somnus* that is sensitive to killing in complement-containing bovine serum.

The present invention also includes methods of immunization using a vaccine
25 comprising *H. somnus* lacking genes for a family of proteins associated with serum resistance. These genes encode immunoglobulin (Ig) binding proteins such as an approximately 120 kDa group of extracellular fibril associated Ig binding proteins and a 76 kDa Ig binding protein present in the outer membrane (Corbeil et al., *Infect. Immun.* 65:4250-4257 (1997)). These Ig binding proteins bind the Fc portion of bovine IgG2.
30 Virulent strains of *H. somnus* bind IgG2 to the surface and it is believed such strains evade immune recognition by the host because critical protective antigens expressed by the pathogen are masked by the bound bovine IgG2. Thus, *H. somnus* organisms that express

decreased amounts of Ig binding proteins because of gene deletion, mutation or by other mechanisms are useful herein for vaccinating cattle. *H. somnus* strains 1P, 129Pt, 130Pfl and 133P (deposited as PTA-600, PTA-601, PTA-602 and PTA-603, with the ATCC) are missing 13.4 kb of DNA, which encodes the 120 kDa group and 76 kDa Ig binding proteins discussed above.

Another feature of *H. somnus* rendering it useful as a vaccine is the expression of a 40 kDa (p40) protective surface antigen (Corbeil et al., *Infect. Immun.* 59:4295-4301 (1991)). Monospecific bovine IgG1 and IgG2 antibody stimulated against such p40 antigen passively protects calves against *H. somnus* induced pneumonia (Gogolewski et al., *Infect. Immun.* 56:2301-2316 (1988)). The antigen is expressed on the surface of *H. somnus* (*id.*) and conserved in all strains tested (*id.*). Furthermore, this p40 antigen cross-reacts strongly with surface exposed antigens of other organisms, including, *P. haemolytica* and *P. multocida* (*id.*). Thus, expression of the p40 surface antigen in *H. somnus* of the vaccine also can protect cattle against infection by other organisms.

Another important feature of a useful vaccine based on gram negative organisms is the avoidance of serious complications often associated with endotoxin from the vaccine. *H. somnus* produces a lipooligosaccharide (LOS) which has endotoxic activity similar to that of *E. coli* J5 LOS (Inzana et al., *Infect. Immun.* 56:2830-2837 (1988)) and pathogenic *H. somnus* organisms that have been previously used as a vaccine are known to be associated with serious inflammation or endotoxic shock (Ellis et al., *Can. Vet.* 38:450-47 (1997)). Thus, a vaccine that sheds less LOS should have reduced toxicity.

In this regard, the present invention provides *H. somnus* organisms that release substantially reduced amounts of endotoxin during growth. The amount of LOS released by *H. somnus* in the vaccine of the present methods is preferably less than that released by virulent strains, more preferably less than 10% of that released by virulent strains and most preferably less than 1% of that released by virulent strains. For example, virulent strain 2336 releases almost 0.04 mg/ml (40 µg/ml) LOS in supernatant at 24 hours of culture (Example 1). Thus, nonvirulent *H. somnus* strains useful as a vaccine of the invention preferably release less than 40 µg/ml LOS, more preferably less than 4 µg/ml LOS, and most preferably less than 0.4 µg/ml of LOS into the culture supernatant during about 24 hours of culture, which includes an exponential growth phase followed by a stationary growth phase.

H. somnus strains 1P, 129Pt, 130Pfl and 133P (deposited as PTA-600, PTA-601, PTA-602 and PTA-603, with the ATCC) release much reduced levels of LOS during log and stationary phases of growth, although these natural isolates have similar amounts of LOS associated with the cell pellet as does the virulent *H. somnus* (e.g. strain 2336, 649 and 8025). Since free endotoxin of *Haemophilus Influenzae* was shown to be more toxic than cell bound endotoxin (Gu et al., *Infect. Immun.* 63:4115-4220 (1995)), a significant reduction in released endotoxin is likely to be important in preventing tissue reactions at the inoculation site and systemic reactions to vaccination that occur frequently with virulent *H. somnus* bacterins.

LOS with complete core sugars undergoes antigenic variation resulting in evasion of host response (Inzana et al., *Infect. Immun.* 60:2943-2951 (1992)). LOS from virulent serum-resistant strains of *H. somnus* undergoes antigenic variation *in vivo* and *in vitro*, but LOS from some serum-sensitive preputial isolates does not undergo antigenic variation, at least *in vitro* (*id.*). Thus, the LOS that remains associated with the organism in serum-sensitive *H. somnus* isolates used in the vaccines of the present invention have the added advantage of providing a more stable antigenic target than LOS associated with virulent strains.

The mechanism by which natural isolates from asymptomatic carriers release less LOS is unknown. Nevertheless, *H. somnus* organisms with this feature can be found by screening natural isolates from healthy cattle. Such organisms can be identified by analyzing culture medium of growing organisms for LOS as described in Example 1 using the silver staining method Tsai-Frasch or by detection of LOS using monoclonal antibody prepared essentially as described in Inzana et al., *Infect. Immun.* 56:2830-2837 (1988)). In addition, a reduction in released endotoxin can be shown in an animal model of endotoxic shock in which live organisms (generally about 10^6 to 10^9 cells) are injected intraperitoneally into mice and endotoxic shock determined by lethality or moribundity.

The *H. somnus* vaccine is preferably administered as an attenuated live vaccine. With live vaccines, the amount of organism in a useful dose is generally less than for killed vaccines. Consequently, live vaccines have the advantage of presenting less endotoxin to the recipient and avoiding some of the associated toxicity, including local tissue reactions and occasionally death. Although administration of a live *H. somnus* vaccine raises concerns of septicemia following multiplication and dissemination, live *H. somnus* that are

sensitive to complement-containing bovine serum do not raise such concerns because the plasma complement of blood should kill these organisms when they reach the blood stream. Organisms lacking genes associated with serum complement resistance and lacking expression of one or more Ig binding proteins are particularly suited for use as a live
5 attenuated vaccine because the encoding DNA is missing from such organisms.

However, administration of vaccines wherein the *H. somnus* organisms are killed also is contemplated herein. The organisms can be killed by methods well known in the art including, for example, by chemical methods such as formalin or by physical inactivation methods such as by heat.

10 A live or killed *H. somnus* vaccine can be administered systemically, or by any other suitable route including, for example, intradermally, intramuscularly, or subcutaneously. In particular, the vaccine can be administered to a mucosal surface such as the nasal, upper respiratory tract or vaginal surface as these surfaces are naturally colonized by *H. somnus*. The vaccine can be administered in a conventional active immunization
15 scheme: single or repeated administration in a manner compatible with the dosage formulation, and in such amount as will be prophylactically effective, i.e. the amount of immunizing *H. somnus* antigen that induces immunity in cattle against challenge by virulent *H. somnus*. Immunity is defined as the induction of a significant level of protection in a population of cattle after vaccination compared to an non-vaccinated group.

20 An attenuated live vaccine which is serum-sensitive is preferably administered by inoculation subcutaneously or on a mucosal surface. This is desirable because the administered organisms are initially viable and can replicate at such sites until they are killed by complement that accumulates during inflammation. Because serum-sensitive strains are killed by complement, they would not survive in complement-containing tissue
25 such as an inflammatory site or in the blood. The ability of an attenuated live vaccine to at least replicate for a short time in the host is generally associated with improved immunity over that obtained with a killed vaccine.

Administration of the vaccine via a mucosal route also has the advantage of eliciting protective IgA as well as IgG antibody. Such antibodies have been elicited by respiratory
30 inoculation of virulent *H. somnus*, resulting in protection against challenge with 10X the original infective dose (Gogolewski et al., *J. Clin. Microbiol.* 27:1767-1774 (1989)).

Vaccine formulations will contain an effective amount of the active ingredient, i.e., *H. somnus* or a preparation thereof, in a pharmaceutically acceptable vehicle, the effective amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends upon factors such as the age, weight and physical condition of the animal considered for vaccination. The quantity also depends upon the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. Effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

Vehicles for the vaccine include, for example, aqueous saline, aqueous buffer, or other known substances. The vehicle also can include other constituents known to increase the activity and/or the shelf life. These constituents may be salts, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers, adjuvants to improve the immune response (e.g. oils, muramyl dipeptide, aluminum hydroxide, saponin, polyanions and amphipatic substances) and preservatives, (e.g. chlorobutanol and benzalkonium chloride).

The vaccine containing *H. somnus* can be tested in vivo for efficacy in animal models or experimental *H. somnus*-induced disease in the natural host. Such models include pneumonia, abortion and septicemia.

Immunity to *H. somnus*-induced pneumonia in cattle can be evaluated in models reported previously (Gogolewski et al., *Infect. Immun.* 55:1403-1411 (1987); Gogolewski et al., *Vet. Path.* 24:250-256 (1987)). In this approach, cattle immunized the vaccine administered as described above are tested for efficacy by administering small doses of *H. somnus* strain 2336 (10^6 - 10^8 CFU) in 2 ml intrabronchially by flexible fiber optic scope or nasotracheal tube to 6-12 week old calves. Transtracheal inoculation of the vaccine also can be used in this model.

Immunity to experimental *H. somnus*-induced abortion can be evaluated in models reported previously (Widders et al., *Infect. Immun.*, 54:555-560 (1986); Corbeil et al., *Infect. Immun.* 55:1381-1386 (1987)). In this approach, pregnant cattle previously immunized with the vaccine administered as described above are tested for efficacy by administering large doses (4×10^{10} CFU) of virulent *H. somnus* (e.g., strain 649) either intravenously or intrabronchially.

Immunity to experimental *H. somnus*-induced septicemia can be evaluated in mice or cattle immunized with vaccine administered as discussed above wherein septicemia is induced by intravenous or intraperitoneal inoculation of virulent organisms in cattle or mice, respectively.

5 *H. somnus* organisms used in the vaccine of the present invention can be genetically modified so as to acquire any of the features described above. For example, *H. somnus* organisms can be modified to express the 40 kDa *H. somnus* surface antigen associated with vaccine protection if the organisms do not express such antigen. Alternatively, an additional gene for the 40 kDa *H. somnus* antigen can be genetically inserted into the
10 organism to enhance the resulting immune response and increase protection. Such a vaccine can induce antibodies against cross reactive surface antigens of *H. somnus*, *P. multocida* and *P. haemolytica* (Corbeil et al., *Infect. Immun.* 59:4295-4301 (1991)). In addition, other *H. somnus* antigen-encoding genes can be genetically inserted into *H. somnus*. Such antigens include, for example, p76, p78, p60, p39 and the like, which
15 provide protection against *H. somnus*-induced disease and some minor cross protection against other *Pasteurellaceae*-induced disease.

The present invention also provides methods of protecting cattle by immunizing with a recombinant multivalent *H. somnus* vaccine that results in protective immunity against disease causing agents other than *H. somnus*. Genes for antigens of other pathogens
20 causing syndromes in cattle also can be used to construct a recombinant multivalent vaccine based on *H. somnus* (e.g., bovine respiratory disease). By this approach, protection that builds upon the cross-protectivity of the *H. somnus* antigens is achieved by using recombinant techniques to express protective antigens from *H. somnus*-related disease-causing organisms such as from other *Pasteurellaceae*. For example, the leukotoxin genes
25 of *P. haemolytica* can be expressed by recombinant methods in *H. somnus* organisms of the vaccine to provide both specific anti-leukotoxin antigen and cross-protective anti-40 kDa outermembrane antigen mediated-protection. Therefore, the vaccine would protect against both *H. somnus* and *P. haemolytica*. Genes for other protective antigens of the *Pasteurellaceae* family of organisms also may be expressed in *H. somnus* organisms to
30 provide a vaccine broadly protective for a group of infections (e.g., bovine respiratory disease caused by *P. haemolytica*, *P. multocida* and *H. somnus*).

To protect against bovine reproductive failure, genes of organisms causing abortion or infertility such as protective surface antigens of *Leptospira interrogans*, *Neospora caninum*, *Tritrichomonas foetus*, and/or *Campylobacter fetus subsp. venerealis*, can be expressed by genetically engineering the *H. somnus* strains discussed above. Other combinations could be used to protect against agents causing septicemia, arthritis, and/or meningoencephalitis.

A multivalent *H. somnus* vaccine also can be engineered to provide protection against bacterial and viral diseases of cattle. For example, protective antigens for viral BRD or BRF diseases of cattle can be expressed in the *H. somnus* organisms of the vaccine.

Such vaccines can comprise *H. somnus* expressing protective viral antigens alone or in combination with other protective bacterial antigens.

Multivalent recombinant vaccines for pneumonia and septicemia can be administered to animals at an appropriate age while a multivalent recombinant vaccine for reproductive failure can be administered to animals at an appropriate time before breeding. Methods for introducing genes into bacteria or deleting/inactivating host genes are well known in the art. Example 2 describes cloning vectors and recombinant DNA strategies for genetically engineering *H. somnus* to express foreign genes and to delete host genes.

EXAMPLES

Example 1:

Analysis of *H. somnus* Strains for Proteins and Endotoxins

This example describes methods for growing *H. somnus* and measuring protein and endotoxin associated with cells and released into the supernatant.

H. somnus organisms were grown in brain heart infusion broth containing 0.1% Tris base and 0.01% thiamine monophosphate by vigorous shaking at 37°C. At various times, a sample of culture was removed and adjusted to 75% light transmission (610 nm) and the cells (CP) were separated from the supernatant (S) by centrifugation. Endotoxin (LOS) and protein antigens (PA) associated with the cell pellet and the supernatant were

analyzed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, respectively.

For LOS detection, cell pellet (CP) and supernatant (S) were digested with RNase followed by proteinase K, samples were run on SDS-PAGE (15% polyacrylamide and 3% urea) and LOS was visualized in the gel by the Tsai-Frasch silver staining method (Tsai et al., *Ann. Biochem.* 119:115-119 (1982)). Quantitation of LOS in the SDS gels was accomplished using LOS standards obtained by extracting LOS from *H. somnus* virulent isolates using a modification of the hot phenol-water method of Westphal (Westphal and Jann, Academic, Press, New York p83-91 (1965)). Standards and experimental LOS samples were evaluated by densitometry using the NIH Image Program, v 1.60.

Proteins were detected by Western blotting essentially as described in Gogolewski et al., *Infect. Immun.* 55:1403-1411 (1987)). Samples of CP and S, solubilized in SDS-PAGE sample buffer, were run on standard Laemmli SDS-PAGE, electrotransferred to nitrocellulose paper and then immunoblotted using convalescent bovine serum (Gogolewski et al., *Infect. Immun.* 55:1403-1411 (1987)) followed by anti-bovine Ig antibody alkaline phosphatase conjugate.

For cell pellets from both virulent and natural isolates, the amount of LOS or PA detected remained constant over time. The release of PA was minimal, increasing slightly over time. However, for virulent strains 2336 and 640, free LOS doubled from early (5 to 6 hrs) to late log phase (10 hrs), reaching about 0.04 mg/ml of S, a value about half that of LOS in the cell pellet. Free LOS in the supernatant doubled again in amount at 24 hrs (the stationary phase).

For the natural isolates from asymptomatic carriers, 129Pt and 1P, S from stationary cultures at 24 hours contained almost no LOS detectable by silver staining of SDS-PAGE gels, although the amount in CP was about the same as for the virulent strains.

Example 2:

Preparation of Genetically Engineered *H. somnus* Vaccine

This example describes recombinant DNA methods for genetically engineering *H. somnus* organisms to express foreign genes or delete selected host genes.

a) Modification and Subcloning of *H. somnus* Genes:

To facilitate subcloning into pLS88Bgl II, the recombinant plasmid pHS139 (Cole et al., *Mol. Microbiol.* 6:1895-1902 (1992); Cole et al., *J. Gen. Microbiol.* 139:2135-2143 (1993)), which expresses the p76 protein was modified in the following manner. pHS139 was digested with *Pvu* II and the 5.5 kb fragment which contained the insert and flanking vector DNA was isolated. *Cla* I linkers were ligated to the 5.5 kb fragment. The ligation was digested with *Cla* I and *Bam*H I and the resulting 5.2 kb fragment was isolated. The plasmid pLS88Bgl II was digested with *Cla* I and *Bgl* II and the 4.6 kb fragment was isolated. The 5.2 kb *Bam*H I/*Cla* I fragment containing the *p76* gene was ligated to the 4.6 kb *Cla* I/*Bgl* II vector fragment of pLS88Bgl II. The ligation was electroporated into *E. coli* strain DH5 α with selection for streptomycin resistance. Plasmid DNA was isolated from selected clones and the presence of the 5.2 kb insert within the 4.6 kb vector was determined by restriction analysis. The recombinant plasmid was designated pJDS160. Subsequently the plasmid pLS88Poly has been utilized for subcloning the gene of the p120 Ig binding protein family (pJDS161). Additionally, the kanamycin gene flanked by *Bam*H I sites has been used to engineer a construct designed to inactivate the gene encoding the p120 Ig binding protein family (pJDS162).

20

b) In Vivo Methylation of Recombinant Plasmids:

Differences in restriction modification can impact the efficiency at which DNA from one bacterial organism is taken up by another. Transformation of recombinant plasmids from *E. coli* into *H. influenzae* suggest this fact and restriction modification was reported as a problem with genetic exchange in *P. haemolytica* (Briggs et al., *Appl. Environ. Microbiol.* 60:2006-2010 (1994)). These observations indicate that prior methylation of recombinant plasmid constructs might overcome difficulties with electroporation of plasmid DNA into *H. somnus*.

30

The restriction modification system of *H. somnus* has not been characterized and while commercially available methylases might protect one or more sites, a much more broad scale protection is desirable. The restriction modification system (including methylation sites) has been characterized for the related species *H. influenzae* and the genetics of this species has been thoroughly investigated. Furthermore, *H. influenzae* genes

cloned in *E. coli* could be transferred back into *H. influenzae* although at a reduced efficiency as compared to *H. influenzae* to *H. influenzae* gene transfer. Thus, recombinant vectors containing *H. somnus* genes could be introduced into *H. influenzae* for methylation and then removed and used for transformation of *H. somnus*.

5 Analysis of the nucleotide sequence of the insert from pHS139 shows 13 potential sites for four *H. influenzae* restriction enzymes (with concurrent methylation sites). *H. influenzae* Rd strain DB117, a recombinant-deficient (*rec-I*) cloning strain (plasmids introduced into the strain are unable to undergo recombination with the chromosome), was selected as a methylation source. All recombinant plasmids were first electroporated into
10 this strain. Recombinant plasmids were isolated after methylation and their identity was confirmed by restriction analysis. While this system was applied to methylation of *H. somnus* genes previously cloned into *E. coli*, this system should be applicable to methylation of cloned genes from varied sources.

c) Conditions for Electroporation of Recombinant Plasmids into *H. somnus*:

15 Recombinant plasmids were electroporated into *H. somnus* under optimized conditions. Strains were grown in brain heart infusion broth supplemented with 0.01 % thiamine monophosphate and 10 % Levinthal Base to an optical density, OD₆₀₀ of 0.600 (+/- 0.100). Cells were chilled on ice for 30 minutes, and then harvested by centrifugation
20 at 4300 X g for 5 minutes at 4°C. *H. somnus* cell pellets were washed twice in 272 mM sucrose buffer with centrifugation for 20 minutes at 4,300 X g for each wash. After the final wash, the cell pellet was suspended in cold 272 mM sucrose buffer to yield a 100 fold increase over the original cell concentration. Cell volumes of 39 µl and DNA concentrations of about 300 ng were used for electroporation.

25 Electroporation of *H. somnus* was at a field strength of 16.0 Kv/cm with a cuvette gap of 1 mm and a resistance of 186 ohms. Reactions after pulsing were diluted to 1 ml with media, chilled on ice for 10 minutes, incubated at 37°C for 1 hour, and plated for selection. Plasmid DNA was isolated from selected clones and the identity was confirmed by restriction digests.

30 Expression of the introduced genes was demonstrated by Western blot analysis of lysates of selected clones. In addition to the electroporation of pJDS160 and consequent expression of the p76 protein in *H. somnus* strain 129Pt, constructs pJDS161 and pJDS162

also have been electroporated into 129Pt. Although conditions for electroporation have been established for *H. somnus* strain 129Pt, conditions may need to be varied for different strains.

5 d) Inactivation of *H. somnus* genes by Deletion/Insertion:

The general approach to gene inactivation involves introduction of the specific gene with a significant portion of the encoding region deleted and replaced with a selectable marker (e.g., kanamycin resistance gene from pLS88PolyKan utilizing flanking
10 multiple cloning sites). Inactivation of the specific chromosomal gene relies on homologous recombination with common DNA flanking the antibiotic resistance marker. After introduction of the modified gene into the target strain by electroporation, homologous recombination with allelic exchange can occur in two forms (i) as fragment with minimal flanking vector DNA, or (ii) as an insert within a suicide vector. With either
15 approach, the introduced genetic elements would not be able to replicate independently in the target strain.

The multiple cloning sites flanking the kanamycin gene present in pLS88PolyKan offers the potential to inactivate specific genes of *H. somnus* to produce avirulent strains or to produce inactivated, selectable genes from different pathogens for recombinant vaccine
20 construction. The use of a fragment for homologous recombination may be more specific for allelic exchange than the suicide vector as shown previously for *H. ducreyi* (Hansen et al., *J. Bact.* 174:5442-5449 (1992)).

The p120 gene encoding an Ig binding protein can be inactivated using this system. The subclone, pHS119, was used as a basis for deletion/inactivation. The plasmid pHS119
25 contains the C-terminal region of the gene encoding the p120 protein family. The *Hind* III insert of pHS119 was ligated into the *Hind* III site of pLS88. The kanamycin gene from pLS88PolyKan with flanking *Bam*H I sites was ligated into the *Bgl* II site of the insert creating pJDS162.

To inactivate the gene encoding the p120 Ig binding protein, the insert with minimal
30 flanking vector DNA is excised from pJDS162, isolated, and electroporated into an *H. somnus* strain expressing the high molecular weight (HMW) Ig binding proteins. Inactivation of the gene encoding the p120 protein occurs through homologous recombination with selection for kanamycin resistance as an indication of allelic exchange.

Kanamycin resistant clones are screened for expression of HMW Ig binding proteins by Western blotting. Integration of the kanamycin resistance gene within the chromosomal gene encoding the p120 protein is demonstrated by Southern blotting.

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The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the preferred embodiments of the compositions, and are not intended to limit the scope of what the inventors regard as their invention. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All publications, patents, and patent applications cited in this specification are incorporated herein by reference as if each such publication, patent or patent application were specifically and individually indicated to be incorporated herein by reference.

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What is claimed is:

1. A method for vaccinating cattle against diseases mediated by infection, comprising administering an effective amount of an *H. somnus* vaccine, wherein the *H. somnus* is susceptible to killing by bovine complement-containing serum.

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2. The method of claims 1, wherein the *H. somnus* releases reduced amounts of endotoxin during growth as compared to virulent *H. somnus*.

3. A method for vaccinating cattle against diseases mediated by infection, comprising administering an effective amount of an *H. somnus* vaccine, wherein the *H. somnus* releases reduced amounts of endotoxin as compared to virulent *H. somnus*.

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4. The method of claim 3, wherein the *H. somnus* is susceptible to killing by bovine complement.

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5. The method of any of claims 1 to 4, wherein the *H. somnus* is live.

6. The method of any of claims 1 to 4, wherein the *H. somnus* is killed.

7. The method of any of claims 1 to 4, wherein the *H. somnus* lacks the expression of one or more immunoglobulin binding proteins expressed by virulent *H. somnus*.

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8. The method of claim 7, wherein the lack of expression of one or more immunoglobulin binding proteins is achieved by the step of genetically engineering *H. somnus* to delete one or more genes encoding the one or more immunoglobulin binding proteins.

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9. The method of any of claims 1 to 8, wherein the *H. somnus* expresses a protective antigen.

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10. The method of claim 9, wherein the protective antigen is a 40 kDa outermembrane protein.

11. The method of any of claims 1 to 10, wherein the *H. somnus* is selected from the group consisting of PTA-600, PTA-601, PTA-602 and PTA-603, deposited with the American Type Culture Collection.

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12. The method of any of claims 1 to 11, wherein the *H. somnus* is genetically engineered to express one or more protective antigens.

13. The method of claim 12, wherein the *H. somnus* is genetically engineered to
10 express one or more protective antigens from pathogens other than *H. somnus*.



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FIG. 1

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